GLUCOCORTICOID-MEDIATED INDUCTION OF CYP3A4 IS DECREASED BY DISRUPTION OF A PROTEIN: DNA INTERACTION DISTINCT FROM THE PREGNANE X RECEPTOR RESPONSE ELEMENT

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ABSTRACT:

CYP3A4 is the most abundant cytochrome P450 (P450) in human liver, comprising approximately 30% of the total liver P450 content. This enzyme has an important role in steroid catabolism and metabolism of foreign compounds, with the majority of pharmaceutical drugs being substrates for CYP3A4. The molecular mechanisms that underlie transcriptional activation of CYP3A4 are complex with many steroid hormone nuclear receptors, including glucocorticoid receptor, pregnant X receptor (PXR), vitamin D receptor, and constitutive androstane receptor, playing roles. Where is this more evident than in the induction of CYP3A4 gene expression by glucocorticoids. CYP3A4 genes lack a consensus glucocorticoid receptor response element and yet are highly induced by classical glucocorticoids such as hydrocortisone and dexamethasone. Recent evidence has demonstrated that glucocorticoids are ligands for the orphan nuclear receptor PXR, and induction of CYP3A genes by glucocorticoids may occur primarily through PXR interactions. In this paper, we present a mutant that disrupts a hepatoocyte-nuclear-factor-3/CCAAT-enhancer binding protein α binding site in the CYP3A4 proximal promoter. This mutation disrupts induction of a reporter gene construct by the glucocorticoids dexamethasone and hydrocortisone; yet induction by the potent PXR ligand rifampicin is unaffected. Such data provides strong evidence that glucocorticoids induce CYP3A4 gene expression both through the established PXR-dependent pathway but also through a PXR-independent pathway.

The cytochrome P450 superfamily is a group of mixed function oxidases, present in both eukaryotes and prokaryotes (Nelson et al., 1996). The enzymes are based on a haem protein skeleton, with each enzyme having a unique substrate-binding site. The super family therefore exhibits a wide substrate profile, and indeed cytochrome P450 enzymes are responsible for the majority of initial metabolism of chemicals, both endogenous and xenobiotic.

In man, the major site of metabolism is the liver. As expected there are a number of cytochrome P450 molecules present in liver, each with a spectrum of specific substrates. The cytochrome P450 3A (CYP3A) family is the most abundant P450 present in human liver, comprising approximately 30% of the total P450 content (Watkins, 1994). In addition, approximately 60% of pharmaceutical drugs currently in use, which are oxidized during metabolism, are substrates for CYP3A enzymes (Cholerton et al., 1992), meaning that this family is of great clinical importance in xenobiotic metabolism in humans. Of the three CYP3A enzymes present in man, CYP3A4 is quantitatively the most abundant, being found in all but one adult liver sample so far screened (Aoyama et al., 1989). Hence, CYP3A4 probably contributes the major CYP3A-mediated metabolism in the population as a whole.

The molecular mechanisms underlying regulation of CYP3A4 gene expression have been studied by several groups, including ourselves. Initially, 1105 bp of proximal promoter was isolated (Hashimoto et al., 1993), and computer analysis showed the presence of several putative transcription factor binding sites including the estrogen receptor, chicken ovalbumin upstream promoter-transcription factor, HNF-4, HNF-5, p53, and octamer transcription factor-1 (Hashimoto et al., 1993). Importantly no consensus glucocorticoid receptor binding site was identified, despite the fact that CYP3A4 is transcriptionally activated by glucocorticoids (Ogg et al., 1999). This raised the possibility of activation via a nonconsensus glucocorticoid responsive unit, as seen in the rat CYP3A4 ortholog CYP3A23 (Huss et al., 1996).

There are three possible mechanisms by which glucocorticoids cause induction of CYP3A4 gene expression. The simplest case, and the one for which there is the least evidence, is that this induction occurs via direct interaction of GR with the CYP3A4 promoter. As mentioned above, no consensus GRE is present in either CYP3A4 or CYP3A23 promoters, suggesting that if such an interaction does occur, it would be via a nonconsensus GRE. Such a scenario cannot be excluded as there is a body of evidence demonstrating DNA-protein interactions of GR with other response elements, including activator protein-1, nuclear factor-κB, and simian virus 40 promoter factor 1 (Bamberger et al., 1996). However, such a mechanism could
not be the sole route of induction as murine GR knock-outs suggest the role of this receptor is nonessential for induction (Schuetz et al., 2000), although such knock-outs could have resulted in up-regulation of other "rescue" pathways not usually seen in vivo.

The synthetic glucocorticoid dexamethasone has been shown to act as a ligand for PXR, the major steroid hormone nuclear receptor controlling expression of CYP3A gene expression, albeit a poor one (Lehmann et al., 1998). As a consensus direct repeat for PXR binding (DR3) is present within the CYP3A23 glucocorticoid responsive unit (Huss et al., 1996), it can be hypothesized that glucocorticoid induction of CYP3A occurs through activation of PXR. Finally, glucocorticoids may interact with GR, which in turn stimulates other transcription factors that interact with the CYP3A promoter. Pascussi and colleagues have demonstrated that dexamethasone produces an increase in the level of the nuclear receptors PXR, CAR, and retinoid X receptor α, possibly through GR-stimulated induction of gene expression (Pascussi et al., 2000a,b). Undoubtedly the in vivo situation is extremely complex, with some, if not all, the above mechanisms occurring.

In the current study, we have characterized a mutation within the promoter region of the human CYP3A4 gene which interrupts a putative complex binding site for both the CCAAT-enhancer binding protein α (C/EBPα) and HNF-3 (Schule et al., 1988). The mutation results in a reduced affinity for protein binding at these sites, as demonstrated by electromobility shift assays. Transactivation assays have been used to show that the observed mutation disrupts the ability of the CYP3A4 promoter to respond to glucocorticoids but does not affect the response to the potent PXR-ligand rifampicin. Such data are the first direct demonstration that glucocorticoids may induce CYP3A4 gene expression through PXR-independent pathways.

Materials and Methods

Chemicals. Dexamethasone, hydrocortisone, and rifampicin were all of cell culture grade and purchased from Sigma-Aldrich (St. Louis, MO). Unless otherwise stated, all other chemicals were of molecular biology grade and obtained from Sigma-Aldrich.

Plasmid. The secretory alkaline phosphatase reporter gene pSEAP pro2 was purchased from CLONTECH (Palo Alto, CA). 301 bp of the CYP3A4 5' flanking region (−301 bp→+7 bp) were engineered in this reporter gene (hereafter termed pWT) by PCR cloning. Directional cloning was carried out using primers with restriction enzyme sites for Acc65I (−301A) and BglIII (+7B) added to the 5′ terminus (underlined), attached as described below. The sequence of the −301 bp to +7B region of the CYP3A4 promoter was cloned and sequenced as described under Materials and Methods. The −301 A→+7 B bp sequence (top and bottom strand) and 35 μg of HepG2 nuclear protein extract. Gray shading represents those areas protected on each strand over the region of interest. Extracts are representative of at least three separate experiments on both strands.

FIG. 1. Sequence characterization of mutant CYP3A4 promoter region and associated DNA-protein interactions.

The entire −301 bp→+7 bp region of the CYP3A4 promoter was cloned and sequenced as described under Materials and Methods. * indicates the site of the point mutation, a T→C transition confirmed in both forward and reverse directions. Underlined regions represent computer-predicted binding sites, determined using the Alabba 2.1 search engine to interrogate the TRANSFAC database. DNase I footprinting experiments were carried out using [γ32P]ATP 5′ end-labeled −301 bp→+7 bp sequence and incubated for 24 h at 37 °C in 5% CO2. Transfection mixtures were prepared on ice, with a standard precipitation formation time of 1 min. Plasmid DNA, pWT, pMUT, or pCMV (empty vector control), was used at a concentration of 12.5 μg/ml transfection mixture (equivalent to 1.5 μg/ml). Twenty-four hours after transfection cells were exposed to xenobiotic, dexamethasone, hydrocortisone, and rifampicin and dissolved in DMSO and used in final concentrations of 5, 10, 25, 50, and 100 μM for dexamethasone and rifampicin and 0.1, 0.5, 1, 5, 10, 25, 50, and 100 μM for hydrocortisone. Xenobiotics were dissolved so that final solvent concentration was 0.1%, and solutions were prepared fresh on the day of dosing. Medium was removed from wells and stored at −20 °C for later assay of SEAP activity. Fresh medium was then added to the wells and xenobiotic solution and solvent controls added as required. Each experimental condition was carried out in eight separate wells. Following 48 h of exposure to xenobiotic of solvent, control medium was removed and stored at −20 °C for later measurement of SEAP activity.

SEAP Activity Determination. Aliquots of cell culture medium (25 μl/well) were transferred into 96-well Optiplates (Canberra Packard, Co, Pangbourne, UK). Endogenous alkaline phosphatase activity was deactivated by heat-treatment of the medium at 65 °C for 30 min. SEAP activity was then assayed using the AURORA system (ICN, Thame, Oxn, UK), according to the manufacturer’s protocol. Chemiluminescent output was measured using a LumiCount automated plate reader (Canberra Packard).

Data Analysis. The relative change in SEAP activity between day 3 (before inducer addition) and day 5 (after inducer addition) was calculated for pWT, pMUT, and pCMV (control, reporter vector minus insert) in the presence and absence of xenobiotic. These measurements allow for the control of variation in cell seeding, transfection efficiency, cytotoxicity, or cell proliferative effects of xenobiotics that might otherwise produce anomalous results.

A specific chemical effect was calculated using the values mentioned above and the statistical significance of this value over solvent control tested as described in Plant et al. (2000). I50 (representing the overall maximal induction produced, encompassing ligand-receptor, receptor-receptor, receptor translocation, and receptor-DNA interactions caused by the inducer) and EC50 (drug concentration required to achieve half maximal induction) values were calculated using nonlinear regression analysis (GraphPad Prism v2.0; GraphPad Software Inc., San Diego, CA), and an indication of the overall effect of the xenobiotic, the overall inductive activity (IA), was calculated by dividing I50 by EC50.

Nuclear Protein Extraction. Nuclear protein extracts were isolated according to the protocol of Dignam et al. (1983). Briefly, HepG2 cells were grown to approximately 90% confluence and then collected by trypsinization. Cells were pelleted by centrifugation (1300g for 5 min) and washed twice with phosphate-buffered saline. After the second wash, cells were resuspended in 5× packed cell volume of ice-cold phosphate-buffered saline. Cells were...
pelleted and resuspended in 2 × packed cell volume of buffer A (10 mM Hepes-KOH, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT). Cells were then left to swell on ice for 10 min before disruption using a Dounce homogenizer and pelleted by centrifugation (20000 g for 15 min). The resulting pellet was resuspended in 0.5 × packed nuclear volume (homogenate volume–supernatant volume) of buffer C (25% glycerol, 20 mM Hepes-KOH, pH 7.9, 1.5 mM MgCl₂, 0.2 mM EDTA, 20 mM NaCl, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride). 0.5 × packed nuclear volume of high salt buffer (buffer C containing 1.2 M NaCl) was then added dropwise with swirling, and the suspension homogenized with a Dounce homogenizer. The resulting homogenate was centrifuged at 16000 g for 30 min, and the supernatant (nuclear protein) aliquoted and stored at −80°C. Protein concentration was determined by a modification of the method of Stoscheck (1990) and integrity assessed by SDS-polyacrylamide gel electrophoresis. Each aliquot was taken through only three freeze/thaw cycles to maintain protein integrity.

DNase I Footprinting Assay. Radiolabeled probe was prepared by initial PCR amplification of the −301 bp→+7 bp region of the CYP3A4 promoter using the primers above. Dephosphorylated amplicons were 5′ labeled with [γ³²P]ATP (110TBq/mmol; Amersham Biosciences UK, Ltd., Little Chalfont, Buckinghamshire, UK) for 60 min at 37°C using T4 polynucleotide kinase (Promega, Chilworth Science ParkSouthampton, UK), and restriction digestion was then carried out to remove one labeled end, creating either labeled-upper or -lower strand probe. This probe was further purified by phenol/chloroform extraction and ethanol precipitation. DNase I footprinting was carried out using the Promega core footprinting system according to the manufacturer’s instructions. Samples were Cerenkov counted to ensure equal loading, heat-denatured, and then separated on a 6% denaturing polyacrylamide gel and opposed to X-OMAT LS film (Kodak Ltd., Hemel Hempstead, Herts, UK) for 16 h.

Electromobility Shift Assay (EMSA). Dephosphorylated oligomers were 5′ labeled with [γ³²P]ATP (10–100 TBq/mmol; Amersham Biosciences UK, Ltd.) for 60 min at 37°C using T4 polynucleotide kinase (Promega, UK). Nonincorporated nucleotides were then removed by ethanol precipitation. All binding reactions were carried out at room temperature (22°C) and comprised binding buffer (20 mM Tris-HCl, pH 7.9, 10 mM KCl, 0.5 mM DTT), 50 μg/ml poly-dI/dC and a 14-μg nuclear protein. The reaction was allowed to proceed for 10 min and then 35 fmol of labeled probe was added (plus unlabeled competitor where appropriate) and allowed to proceed for a further 30 min. Samples were then separated on a 4% nondenaturing polyacrylamide gel and opposed to X-OMAT LS film for 16 h. Oligomers used for EMSA assays are as described below, with the alternate base underlined,

Wild-type, TTGATTGAGTTGTTTATGATACCT
Mutant, TTGATTGAGTTGTTTATGATACCT

EMSA Quantitation. EMSA reactions were exposed until all bands were within the linear range of the film and then bands quantified using video-based computerized densitometry on an minimal clinically important difference image analysis system (Imaging Research, Ontario, Canada).

Results

Mutation Characterization. Both the promoter sequences (wild-type and HNF-3/CEBPα mutant) were confirmed by automated sequencing (PE ABI 373; University of Surrey Sequencing Facility) following cloning into the pSEAP2pro plasmid. As shown in Fig. 1, the mutation causes a T→C transition in the DNA sequence at position −190 bp. Binding sites for transactivating factors were assigned using the Alabia 2.1 (http://wwwitl.com/innsbruck/∼grabe/alabia/2/) to search the TRANSFAC database (http://www. transfac.gbf.de/transfac; Wingender et al., 1996). These assignments are shown in Fig. 1. DNase I footprinting studies demonstrated that DNA-protein interactions occurred at the computer-predicted sites (data not shown).

DNase I Footprinting Assay. To examine how this mutation affected binding of transactivating factors to the CYP3A4 promoter, a DNase I footprinting assay was carried out using HepG2 nuclear protein extract, to identify the areas of DNA-protein interaction within this region. Figure 2 shows the DNA footprint of the top strand of wild-type sequence when exposed to nuclear protein extract from uninduced HepG2 cells. Previous experiments have demonstrated that the footprint obtained with HepG2 nuclear proteins is qualitatively identical to that observed with nuclear proteins isolated from human liver (data not shown). It can clearly be seen that several protected footprints occur and these correspond to the computer predicted binding sites for PXR and HNF-3/CEBPα. DNase I footprinting carried out on the mutant sequence with uninduced HepG2 nuclear extract showed identical binding over the region investigated showing that the point mutation does not cause a qualitative change in binding at the HNF-3/CEBPα interaction site (data not shown).

Electromobility Shift Assay. To investigate whether the point mutation caused a quantitative change in binding, as opposed to a qualitative one, EMSA was carried out using oligomers covering the mutation site, as described under Materials and Methods. Binding of HepG2 uninduced nuclear extract to the wild-type oligomer was competed with either an excess of unlabeled wild-type or mutant oligomer. As can be seen from Fig. 3, the mutant oligomer has a significantly weaker competitive effect than the wild-type oligomer, demonstrating that binding of nuclear proteins to the complex HNF-3/CEBPα site is at a higher affinity in the wild-type sequence as opposed to the mutant sequence. Hence, a clear quantitative difference was observed in the binding of nuclear protein to the wild-type and mutant sequences.

Functional Analysis. To investigate the functional impact of the mutation on expression of the CYP3A4 gene, secretory alkaline phosphatase reporter gene constructs were made containing the region −301 bp→+7 bp, from both wild-type and the mutant variation. Experiments were carried out in this short region of the CYP3A4-flanking DNA, as this removed possible complication due to transactivating factor cross talk, and also corresponds to the region previ-
ously defined as the basal transcription unit by Goodwin and colleagues (Goodwin et al., 1999a). The reporter gene constructs were tested over a full concentration response curve with the glucocorticoid hydrocortisone, its synthetic analog dexamethasone, and the potent PXR-ligand rifampicin. Figure 4 shows the result of these experiments. All three compounds produced a concentration-dependent, statistically significant, induction of the pWT reporter gene construct, demonstrating that this region is sufficient for, at least partial, induction of the CYP3A4 gene. However, for the pMUT mutant reporter gene, only rifampicin produced the same profile observed with the pWT construct. For both dexamethasone and hydrocortisone, the observed EC50 remained the same as that seen using pWT, but the maximal induction observed (I max) was reduced by 29 and 57%, respectively, compared with pWT, leading to corresponding decreases in IA values (Fig. 4).

Discussion

The use of reporter gene constructs is becoming an increasingly important tool for dissection of the molecular mechanisms of gene induction by xenobiotics. Many labs, including ours, have developed such reporter gene systems to study the response of the CYP3A4 promoter to exposure to xenobiotics. Our original system relies upon the ~1k bp region of the proximal promoter originally cloned by Hashimoto et al. (1993). Using this system, we have demonstrated induction of the CYP3A4 gene by both xenobiotics (Ogg et al., 1999) and endogenous compounds (El-Sankary et al., 2000). In the current study, we have used a smaller region of the proximal promoter, covering the first 301 bp. Previously, Barwick and colleagues (Barwick et al., 1996) have demonstrated that 179 bp of CYP3A4 proximal promoter is the minimum required to achieve transcriptional activation in response to a number of xenobiotics in both rat and rabbit hepatocytes, when linked to a heterologous promoter. In comparison, Goodwin et al. (1999b) demonstrated 362 bp of CYP3A4 proximal promoter, linked to the endogenous CYP3A4 enhancer element the xenobiotic responsive enhancer module to be able to act as a xenobiotic responsive region when transfected into HepG2 cells. We now confirm this observation in HepG2 cells, showing 301 bp of CYP3A4 proximal promoter to be sufficient to act as basal transcription unit, and mediate induction of the CYP3A4 reporter gene by compounds previously shown to activate the 1105 bp proximal promoter reporter gene [rifampicin, dexamethasone, hydrocortisone, and estrogen; data not shown for estrogen (Ogg et al., 1999)]. It is of interest to note that the EC50 value obtained by Goodwin and colleagues is significantly less than that observed in this study (2 versus 12 μM). Such a difference probably reflects the difference between a system using the native PXR enhancer (xenobiotic responsive enhancer module) as compared with a heterologous promoter. However, it should be noted that we have previously demonstrated that cotransfection with expression plasmids for hGR and hPXR results in a lowered observed EC50 of 2 μM, in concordance with the observations of Goodwin (El-Sankary et al., 2001). Such data emphasizes the importance of the receptor complement within a given cell system in determining the overall effect. All experiments in the current study were carried out in basal HepG2 cells (i.e., without additional receptor expression plasmids). To ensure that an excess of any single receptor did not cause ligands to act through pathways they do not activate at physiological...
concentration of receptors. Such an approach has been validated by our previous demonstration of such a basal system to respond to a wide range of CYP3A4 gene transcriptional activators. Furthermore, we have generated a single base-pair mutant of the CYP3A4 gene that demonstrates an altered response to glucocorticoids but no alteration in response to rifampicin. This is of particular interest as recent evidence has suggested that glucocorticoid induction of the CYP3A4 gene is regulated not via direct interaction of GR with the CYP3A4 promoter but through activation and up-regulation of the steroid hormone nuclear receptor PXR (Pascussi et al., 2000a). Our data suggests this latter hypothesis to be incomplete, as the mutation described herein affects glucocorticoid-mediated gene expression but not rifampicin-mediated, the latter representing a classical PXR ligand.

Following demonstration of the functional effect of this artificial mutant, we examined the DNA-protein interactions behind this effect. DNase I footprinting experiments showed that a DNA-protein interaction did occur at the mutation site, and computer analysis suggested this to be a complex HNF-3/CEBP/H9251 binding site. In addition to demonstrating the interaction, footprinting studies showed no qualitative alteration in nuclear protein binding at this site in the mutant, compared with the wild-type sequence. However, electromobility shift assays showed that nuclear protein binding at the mutant sequence was at a lower affinity than at the wild-type sequence. Such a lowered affinity is consistent with the functional data and suggests that the putative HNF-3/CEBP/H9251 binding acts as a positive regulator of glucocorticoid-mediated regulation of CYP3A4 gene expression.

The role of C/EBP/H9251 in regulating CYP3A4 gene expression has previously been investigated, demonstrating that C/EBP/H9251, along with D element binding protein was capable of increasing the basal expression of a 169 bp fragment of the CYP3A4 promoter (Ourlin et al., 1997). Whereas our observed mutation lies outside the core C/EBP/H9251 recognition site, effects on it cannot be excluded as several studies have shown that nucleotides immediately 5’ to a recognition sequence also play a role in transcription factor binding (Juge-Aubry et al., 1997; Osada et al., 1997; Driscoll et al., 1998). C/EBP/H9251 could potentially act as a bridging factor for interaction of other transcription factors (e.g., GR) with the basal transcription machinery, and it is well established that CBP/p300 interacts with C/EBP/H9251 in this role (Kino et al., 1999). An alternative activation route is through interaction with the HNF-3 portion of this complex binding site. HNF-3 has been implicated in the regulation of the tyrosine aminotransferase gene, a classically glucocorticoid-induced gene. Roux and colleagues (Roux et al., 1995) clearly demonstrated that

**Fig. 4.** Concentration-dependent activation of the wild-type and mutant CYP3A4 reporter gene constructs by xenobiotics and endogenous steroids.

Reporter genes containing 308 bp of the CYP3A4 promoter (~301 bp to +7 bp) were constructed for the published sequence (pWT) and HNF-3/CEBP/H9251 mutant (pMUT) as described under Materials and Methods. These were transiently transfected into HepG2 cells and exposed to a range of concentrations of rifampicin, dexamethasone, or hydrocortisone for 48 h. Alkaline phosphatase activity was measured before and after dosing and the specific chemical effect calculated. Values were then plotted on a lin-log graph and maximal effect, EC_{50}, and inductive ability (IA, I_{max}/EC_{50}) calculated using GraphPad prism PC software. Error bars show compound variation from 8 wells/experimental point, and statistical significance was calculated as described under Materials and Methods (*, P < 0.05). Data are representative of two separate experiments.
HNF-3, acting via complex, overlapping recognition sites, is capable of controlling the magnitude of TAT gene expression to glucocorticoids. Hence, HNF-3 could act as a controller of non-PXR-mediated glucocorticoid-induced induction of the CYP3A4 gene, producing an increased induction above that seen with PXR alone.

We would therefore propose that regulation of CYP3A4 gene expression by glucocorticoids involves a second site within the CYP3A4 proximal promoter besides the PXRE. This site may function independently of or as a modulator of the PXRE-mediated induction. This induction most likely occurs via activation of GR, followed by one of two possible activation routes. First, binding of GR to a non-consensus GRE may occur. It is interesting to note that such interactions have previously been demonstrated at specificity peptide 1 (Sp1) sites (Bamberger et al., 1996); computer and DNase I footprinting analysis suggest two putative simian virus 40 promoter factor 1 sites are present within the −301 bp → +7 bp region of the CYP3A4 promoter (data not shown). GR binding to the promoter could then cause an increase in binding of the basal transcriptional machinery, with C/EBPα and CBP/p300 acting as established-bridging molecules or HNF-3 as a regulator of the magnitude of effect. An alternative route would be through indirect action of GR, through induction of the factors (HNF-3 and/or C/EBPα) binding to this region of the proximal promoter. GR has been previously implicated in the induction of other receptors involved in glucocorticoid-mediated induction, specifically CAR, retinoid X receptor α, and PXR (Pascussi et al., 2000a,b).

In conclusion, we have demonstrated that expression of this clinically important gene is tightly regulated by endogenous steroids, emphasizing the importance of its role in steroid homeostasis. In addition, we hypothesize that this regulation occurs not only through the previously demonstrated activation of the steroid hormone receptor PXR, the major controller of CYP3A4 gene expression, but via a PXR-independent pathway. This pathway involves HNF-3 and/or C/EBPα binding and, we hypothesize, binding of GR at a non-consensus GRE. Such data are extremely important in understanding the way this enzyme is regulated by the body and how clinical intervention may affect this.

References


